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Evolution of Developmental Control Mechanisms

Development and evolution of the lateral plate mesoderm: Comparative analysis of amphioxus and lamprey with implications for the acquisition of paired fins

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ABSTRACT

Possession of paired appendages is regarded as a novelty that defines crown gnathostomes and allows sophisticated behavioral and locomotive patterns. During embryonic development, initiation of limb buds in the lateral plate mesoderm involves several steps. First, the lateral plate mesoderm is regionalized into the cardiac mesoderm (CM) and the posterior lateral plate mesoderm (PLPM). Second, in the PLPM, *Hox* genes are expressed in a collinear manner to establish positional values along the anterior–posterior axis. The developing PLPM splits into somatic and splanchnic layers. In the presumptive limb field of the somatic layer, expression of limb initiation genes appears. To gain insight into the evolutionary sequence leading to the emergence of paired appendages in ancestral vertebrates, we examined the embryonic development of the ventral mesoderm in the cephalochordate amphioxus *Branchiostoma floridae* and of the lateral plate mesoderm in the agnathan lamprey *Lethenteron japonicum*, and studied the expression patterns of cognates of genes known to be expressed in these mesodermal layers during amniote development. We observed that, although the amphioxus ventral mesoderm posterior to the pharynx was not regionalized into CM and posterior ventral mesoderm, the lateral plate mesoderm of lampreys was regionalized into CM and PLPM, as in gnathostomes. We also found nested expression of two *Hox* genes (*LjHox5i* and *LjHox6w*) in the PLPM of lamprey embryos. However, histological examination showed that the PLPM of lampreys was not separated into somatic and splanchnic layers. These findings provide insight into the sequential evolutionary changes that occurred in the ancestral lateral plate mesoderm leading to the emergence of paired appendages.

Introduction

Paired appendages are one of the most successful innovations in the history of vertebrates. Evolution of paired appendages allowed ancient vertebrates to develop sophisticated behavioral and locomotive patterns. According to fossil records, the first pairs of fin-like structures seem to have been acquired in the lineage of ancestral agnathans (Coates, 1994; Janvier, 1996). The amphioxus, a living cephalochordate, and the lamprey, a living limbless vertebrate agnathan, are thought to have diverged prior to the emergence of paired fins (Donghue et al., 2000). Although amphioxus is not the closest sister group to vertebrates, its genome structure and morphology are thought to be similar to those of ancestral chordates (Putnam et al., 2008). Likewise, the lamprey morphology has been stable for 360 million years (Gess et al., 2006). Thus, these two animals could potentially serve as models for developmental comparisons to obtain insights into the evolutionary changes that eventually resulted in acquisition of paired fins.

In gnathostome vertebrates, initiation of vertebrate fin/limb buds in the lateral plate mesoderm involves multiple steps during embryonic development. The lateral plate mesoderm is first subdivided into the cardiac mesoderm (CM) and the posterior lateral plate mesoderm (PLPM), which includes the presumptive paired fin/limb-forming fields. In the PLPM, *Hox* genes are expressed in a collinear fashion to establish positional values along the anterior–posterior axis. The lateral plate mesoderm then thickens and splits into somatic and splanchnic layers. In the CM, cardiac progenitor cells from the splanchnic layer fuse at the ventral midline to form the myocardium; in the PLPM, the expression of limb initiation genes appears in the fin/limb-forming fields of the somatic layer to induce fin/limb buds (Logan, 2003; Ruvinsky and Gibson-Brown, 2000).

Recent studies in zebrafish and mouse embryos suggest that retinoic acid signaling delimits the size of the CM to provide an environment that is permissive for induction of the forelimb field in the PLPM (Waxman et al., 2008; Zhao et al., 2009). This finding suggests that the regionalization of the lateral plate mesoderm into the CM and PLPM is a prerequisite for establishment of the forelimb

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field. In the gnathostomes, the homeobox transcription factor gene *Nkx2.5* is expressed in the CM (Deimling and Drysdale, 2009; Lints et al., 1993). In the cephalochordate amphioxus, on the other hand, the ventral mesoderm expresses *AmphiNk2-tin*, the ortholog of *Nkx2.5*, and is thought to correspond to the lateral plate mesoderm of gnathostomes (Holland et al., 2003; Kozmik et al., 2001). In limbless agnathan lampreys, the CM is found in the anterior-most part of the lateral body wall (Kokubo et al., 2010). In the lamprey embryo, a single layer of free cells, observed posterior to the liver and the heart, is assumed to be composed of lateral plate-derived cells (Damas, 1944; Shipley, 1887). However, no molecular evidence has been available to support these observations, and the distribution of the PLPM in amphioxus and lamprey remains unclear.

In gnathostomes, collinear expressions of *Hox* genes have been suggested to pattern the PLPM along the anterior–posterior axis and determine the position of fins/limbs (Cohn et al., 1997; Nowicki and Burke, 2000). However, it remains uncertain when and how vertebrates acquired *Hox* expression in the lateral plate mesoderm during evolution. Previous studies have shown that *Hox* genes are not expressed in the so-called somites of amphioxus, which give rise to the ventral mesoderm (Holland et al., 1992; Wada et al., 1999), suggesting that acquisition of *Hox* expression occurred in the ancestral lateral plate mesoderm after the divergence from cephalochordates. On the other hand, *Hox* gene transcripts have not been clearly observed in the lateral plate mesodermal cells of agnathan lampreys (Takio et al., 2007; Takio et al., 2004).

In gnathostomes, cells of the PLPM proliferate and the thickened PLPM then becomes divided into somatic and splanchnic layers. Previous studies with transgenic mice have shown that the forkhead transcription factor *Foxf1* plays crucial roles in the separation of the lateral plate mesoderm into the somatopleure and splanchnopleure by creating the coelom cavity (Mahlapuu et al., 2001). In *Foxf1*-deficient mouse embryos, the proliferation rate of lateral plate mesoderm cells is low, and the underdeveloped lateral plate mesoderm fails to form a coelom (Mahlapuu et al., 2001). Furthermore, depletion of *Foxf1* results in ectopic expression of *irx3*, a marker for the somatopleure, throughout the lateral plate mesoderm (Mahlapuu et al., 2001). These findings suggest that *Foxf1* promotes growth and differentiation of the lateral plate mesoderm and leads to its subdivision into somatic and splanchnic layers by forming the coelom cavity. In the somatic layers of the PLPM, expression of the T-box transcription factor *Tbx5* is found in the fin/limb-forming fields (Gibson-Brown et al., 1996; Isaac et al., 1998; Logan et al., 1998), where it activates *Fgf10* expression in the mesoderm, leading to fin/limb bud initiation (Agarwal et al., 2003; Ahn et al., 2002; Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999; Takeuchi et al., 2003). Orthologous genes for *Tbx4* and *Tbx5* have been identified in the genomes of both amphioxus and lampreys (Kokubo et al., 2010; Ruvinsky et al., 2000). Amphioxus *Tbx4/5* is expressed in the ventral mesoderm posterior to the pharynx (Horton et al., 2008). Interestingly, amphioxus Tbx4/5 has the potential to initiate limb outgrowth when expressed in the PLPM of mouse embryos (Minguillon et al., 2009). Although the developmental process of the lateral plate mesodermal cells in lampreys has not yet been described well (Damas, 1944; Shipley, 1887), expression of *Tbx4/5* has been reported in the putative CM (Kokubo et al., 2010), but not in the PLPM.

Here we investigated the development of the lateral plate mesoderm of cephalochordate amphioxus and agnathan lampreys. We show that the amphioxus ventral mesoderm posterior to the pharynx is not molecularly specified into the CM and posterior ventral mesoderm, whereas the lamprey lateral plate mesoderm is likely to be specified into the CM and PLPM. We also show that lampreys appear to have acquired *Hox* expression domains in the PLPM. However, unlike gnathostomes, the underdeveloped PLPM of lamprey embryos does not split into somatic and splanchnic layers. These results allow us to speculate about the evolutionary implications of the acquisition of paired appendages in the lateral plate mesoderm of ancestral vertebrates.

Materials and methods

Biological materials

Adult Florida amphioxus (*Branchiostoma floridae*) were collected in Tampa Bay, Florida, USA, during the summer breeding season. Eggs and sperm were collected from adults and used for fertilization in vitro, and embryos were raised at 25 °C (Holland and Yu, 2004). Adult male and female lampreys (*Lethenteron japonicum*) were purchased from the Ebetsu Fishery Cooperative, Hokkaido, Japan, during the breeding season (early June). Spawning was induced and embryos were reared to the desired developmental stages at 16 °C in 10% Steinberg's solution (Steinberg, 1957). Lamprey embryos were staged according to Tahara's staging of *Lampetra reissneri* (Tahara, 1988), a species closely related to *L. japonicum*. For in situ hybridization, embryos were fixed overnight in 4% paraformaldehyde in 0.1 M phosphate-buffered saline (PBS), dehydrated in a graded methanol series, and stored in 100% methanol at −20 °C.

Gene isolation and phylogenetic analysis

Total RNA from lampreys was extracted from stage 21–30 embryos using an RNeasy kit (Qiagen, Hilden, Germany). cDNA was synthesized by reverse transcription and used as a template for PCR. We used primers designed based on the nucleotide sequence of a putative *Petromyzon marinus* ortholog found in the genome assembly PMAR3 (http://pre.ensembl.org/Petromyzon_marinus/Info/Index) provided by the Genome Sequencing Center at Washington University School of Medicine (WUGSC): *LjHandA*, 5′-TGGGAAGAAGAGCGCGACAG-3′ and 5′-CCGCTCGAATGTTATGTTATGATTATG-3′; *LjMyb*, 5′-AACGATTGGTGGAGAAACATGG-3′ and 5′-TTTATGCGCTGCTCTCTATCTG-3′; *LjFgf7/10/22*, 5′-ATACTGGAGATCACATCGGTGGAC-3′ and 5′-CTCGAGATTGGCAGGTGGGTAC-3′; *LjFoxF1/2*, 5′-TGACCATGGAGGTGTCTTCAAG-3′ and 5′-CGCCTGACATTTGAGCCTGAAC-3′; *LjIrx1/3*, 5′-GGACTGCAGCACTCGTCTTTCC-3′ and 5′-GTCGATGTTCTCCAAGTCGATC-3′. The partial coding sequences for *LjMyb* (720 bp), *LjFgf7/10/22* (365 bp), *LjFoxF1/2* (413bp), and *LjIrx1/3* (453 bp) have been submitted to GenBank under accession numbers HQ425629, HQ425630, JN019796 and JN019797, respectively. Phylogenetic analysis was used to confirm the orthology assignment of newly identified amphioxus and lamprey genes (Fig. S1). Amino acid sequences were aligned using CLUSTALW (Thompson et al., 1994). Unalignable regions were excluded from analysis. The HLH (helix–loop–helix) and N-terminal domains of Hand, two SANT (switching-defective protein 3, adaptor 2, nuclear receptor co-repressor, transcription factor IIIB) domains of Myb, and a C-terminal domain of Fgf, a forkhead domain of Fox and the homeodomain of Irx were used for each phylogenetic analysis. The neighbor-joining (NJ) phylogenetic trees of amino acid datasets were constructed using the Kimura model (Kimura, 1980). Bootstrapping was carried out using 1000 replicates to estimate the degree of support for internal branches. GenBank accession numbers for the phylogenetic reconstruction are: Human_Hand1, NP_004812.1; Mouse_Hand1, NP_032239.1; Frog_Hand1, NP_001079128.1; Chick_Hand1, NP_990296.1; Mouse_Hand2, NP_034532.3; Zebrafish_hand2, NP_571701.2; Frog_Hand2, NP_001107665.1; Chick_Hand2, NP_990297.1; Human_Hand2, NP_068808.1; Fly_hand, NP_609370.2; Human_Twist1, NP_000465.1; Human_Twist2, NP_476527.1; Chick_c-Myb, NP_990637.1; Frog_c-Myb, NP_001081768.1; Frog_a-Myb, NP_001081179.1; Mouse_a-Myb, NP_032677.2; Human_a-Myb, NP_001073885.1; Chick_a-Myb, NP_990563.1; Human_c-Myb, AAA52030.1; fruitfly_Myb, NP_511170.1; Human_b-Myb, NP_002457.1; AmphiMyb XP_002591806.1; Mouse_b-Myb, NP_032678.1; Chick_b-Myb, NP_990649.1; Human_SNAPC4-001, ENST00000298532; Human_FGF10, NP_004456.1; Chick_Fgf10, NP_990027.1; Zebrafish_fgf10a,

NP_878290.1; Mouse_Fgf10, NP_032028.1; Human_FGF7, NP_002000.1; Chick_Fgf7, NP_001012543.1; Mouse_Fgf7, NP_032034.1; Zebrafish_fgf7, NP_001007762.1; Mouse_Fgf22, NP_075793.1; Zebrafish_fgf22, NP_001035184.1; Human_FGF22, NP_065688.1; Frog_Fgf22, NP_001137396.1; Human_Fgf16, NP_003859.1; Human_Fgf20, NP_062825.1; Human_Fgf9, AAH69692.1; Human_Fgf1, NP_001138406.1; Chick_Irx2, NP_001025507.1; Zebrafish_Irx2, AAH65681.1; Zebrafish_Irx6, NP_001018869.1; Chick_Irx6, XP_001234059.1; Amphioxus_IrxA, ACI49802.1; Human_Irx6, NP_077311.2; Mouse_Irx6, NP_071873.2; Human_Irx4, NP_057442.1; Mouse_Irx4, NP_061373.1; Chick_Irx4, NP_001001744.1; Zebrafish_Irx4, emb|CAK10871.1; Mouse_Irx3, NP_032419.2; Zebrafish_irx3a, NP_571342.1; Chick_Irx3, AAD55977.1; Human_Irx1, NP_077313.3; Chick_Irx1, NP_001025509.1; Human_Irx3, NP_077312.2; Mouse_Irx1, NP_034703.2; Zebrafish_Irx1, NP_997068.1; Amphioxus_IrxC, ACF10240.1; Mouse_Irx5, EDL11086.1; Zebrafish_Irx5, NP_001038692.1; Amphioxus_IrxB, gb|ACF10239.1; Chick_Irx5, XP_001234101.1; Human_Irx5, NP_005844.4; Mouse_Irx2, NP_034704.1; Human_Irx2, NP_150366.1; Frog_FoxA1, NP_989419.1; Zebrafish_FoxA1, AAH56569.1: Human_FoxA1, EAW65844.1; Mouse_FoxA1, gb|AAH96524.1; Human_FoxH1, NP_003914.1; Mouse_FoxH1, NP_032015.1; Zebrafish_FoxH1, NP_571577.1; Frog_FoxH1, NP_001017084.1; Frog_FoxQ1, gb|ABA39837.1; Zebrafish_FoxQ1, NP_998072.1; Human_FoxF2, NP_001443.1; Zebrafish_FoxF2, NP_001077284.1; Human_FoxQ1, NP_150285.3; Mouse_FoxQ1, NP_032265.3; Mouse_FoxF2, NP_034355.2; Frog_FoxF2, NP_001093702.1; Human_FoxF1, NP_001442.2; Mouse_FoxF1, NP_034556.1; Zebrafish_foxf1, NP_001073655.1; Frog_FoxF1, NP_001039226.1; Amphioxus_FoxF.

In situ hybridization and histological analysis

Amphioxus *Nk2-tin*, *Hand*, *Myb*, *IrxC*, and *FoxF* sequences were retrieved from a cDNA resource for the cephalochordate amphioxus *B. floridae* (Yu et al., 2007; http://amphioxus.icob.sinica.edu.tw/). Amphioxus EST clones were kindly provided by the Academia DNA Sequencing Center, National Institute of Genetics in collaboration with the Department of Zoology, Graduate School of Science, Kyoto University. *AmphiNk2-tin* (2100 bp) and *AmphiMyb* (697 bp) were amplified from an amphioxus EST clone (bflv37m11 and bfga014g08, respectively) using primers hybridizing to the published sequence (Holland et al., 2003) or M13F, R, and subcloned into pGEM-Teasy (Promega). Partial *AmphiFoxF* (830 bp; bfne015j03) were amplified from an amphioxus EST clone (bflv37m11) using primers hybridizing to the published sequence (Holland et al., 2003) or M13F and M13R, and subcloned into pGEM-Teasy (Promega). *AmphiHand* EST clone (492 bp; bfga46k20) and *AmphiFoxF* (830 bp; bfne015j03) were excised with *Hin*dIII/*Bam*H1 and *Nco*1/*Eco*R1, respectively, and cloned into pBluescript SK$^-$. These plasmids were used as templates for riboprobe synthesis. The nucleotide sequences of *AmphiHand*, *AmphiNk2-tin* and *AmphiMyb* were deposited in the GenBank database under the accession numbers: HQ605708, HQ605709 and JN034593, respectively. Lamprey riboprobes for *LjHandA* (BAJ05622), *LjTbx4/5* (FJ905042), *LjTbx20* (FJ905044), *LjFgf8* (AB071892), *LjHox5i* (AB125276), and *LjHox6w* (AB125275) were synthesized as described (Kokubo et al., 2010; Kuraku et al., 2010; Shigetani et al., 2002; Takio et al., 2004). Amplified *LjFgf7/10/22* and *LjMyb* were cloned into pGEM-Teasy and used as templates for riboprobe synthesis.

For amphioxus embryos, whole-mount in situ hybridization was performed using a modified protocol in which RNase treatment steps were omitted (Holland et al., 1996). For lamprey embryos, whole-mount in situ hybridization was performed as described (Murakami et al., 2001). Stained lamprey embryos were bleached in 50% and 100% ethanol in PBT (PBS with 0.1% Tween 20), washed several times in PBT, and cleared in 50% glycerol in PBT. Selected amphioxus and lamprey embryos were transferred into 20% sucrose in PBS, embedded in 7.5% gelatin in 15% sucrose, and cut into ~6- to 8-μm-thick cryosections.

For histological analysis, stage 23 and 27 lamprey embryos were embedded for cryosectioning as described above. Stage 30 lamprey embryos were dehydrated in ethanol and acetone, embedded in Technovit 8100 resin (Heraues-Kulzer, Wehrheim, Germany), and sectioned with a FINTEC microtome (Leica). Sections were stained with 0.01% toluidine blue O (Sigma Aldrich) in 1% NaCl, pH 2.3. All sections were cleared in 1:2 (v/v) benzyl alcohol/benzyl benzoate.

RT-PCR

The mesoderm overlying the yolk of stage 21 lamprey embryos, and the PLPM of stage 25 and 27 lamprey embryos were isolated by dissection. Total RNA was extracted from dissected embryos using the RNeasy Mini kit (Qiagen). To remove genomic DNA, each RNA sample was treated with RNase-free DNaseI (Takara). The RNA was used as a template for synthesizing cDNA using AMV Reverse Transcriptase (Promega). We used primers designed based on the nucleotide sequence of Petromyzon marinus 18S ribosomal RNA (GenBank accession number: M97575.1). The following PCR primers for 18S ribosomal RNA and *LjFoxF1/2* were

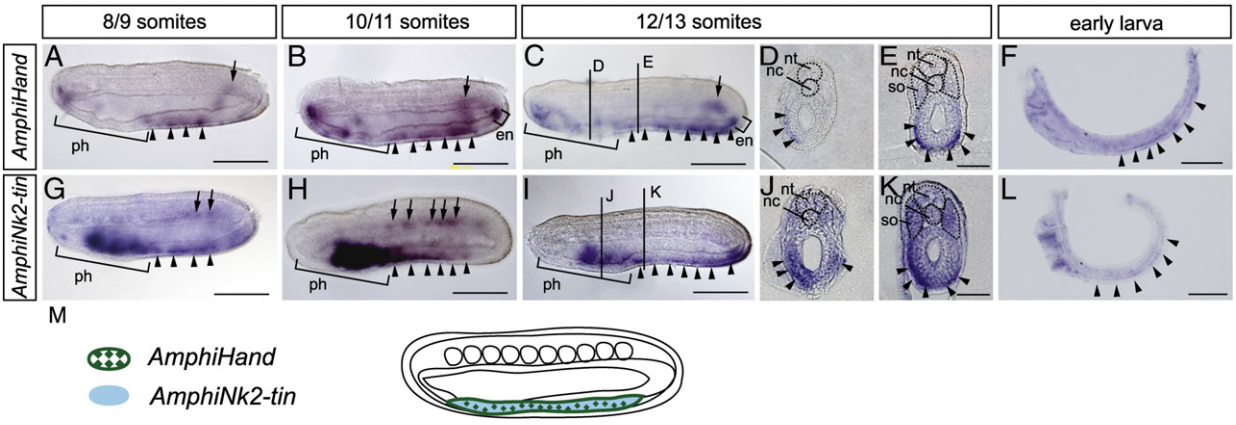


Fig. 1. Expression of *AmphiHand* and *AmphiNk2-tin* in the ventral mesoderm of late neurula amphioxus embryos. (A–C, F–I, L) Lateral views of embryos at the 8/9-, 10/11-, 12/13-somite and early larval stages. Anterior to the left, dorsal to the top. (D, E, J, K) Transverse sections at the 12/13-somite stage where indicated in (C, I). (A–F) Expression of *AmphiHand*. Arrowheads indicate the distribution of *AmphiHand* transcripts in the ventral mesoderm. Arrows indicate the invaginating *AmphiHand*-positive ventral mesoderm. (G–L) Expression of *AmphiNk2-tin*. Arrowheads indicate the distribution of *AmphiNk2-tin* transcripts in the ventral mesoderm. Arrows indicate somites expressing *AmphiNkx2-tin*, as previously described (Holland et al., 2003). en, endoderm; nc, notochord; nt, neural tube; ph, pharynx; so, somites. Scale bars: 100 μm in A–C, E, F–H, J. 25 μm in D, E, J, K. (M) Schematic model of the expression patterns of *AmphiHand* and *AmphiNk2-tin* in the late neurula. Transcripts of *AmphiHand* (green dots) and *AmphiNk2-tin* (light blue) are co-localized in the ventral mesoderm in the late neurula.

used for amplification: 18S ribosomal RNA, 5′-ATTCTAGAGCTAATACATGC-3′ and 5′-ACCCGTGGTCACCATGGTAG-3′; *LjFoxF1/2*, 5′-CCTCATCGTCATGGCGATCC-3′ and 5′-GGTCGATGGTCCAGTAGTGTC-3′.

Results

Establishment of the cardiac mesoderm and posterior lateral plate mesoderm

During development of zebrafish and mouse embryos, regionalization of the lateral plate mesoderm into the CM and PLPM is thought to be an indispensible step for establishment of the pectoral fin/forelimb fields (Waxman et al., 2008; Zhao et al., 2009). Thus, regionalization of the lateral plate mesoderm into the CM and PLPM may also have been a prerequisite for acquisition of paired fins in vertebrates.

To gain insight into the evolution of the lateral plate mesoderm, we first examined the expression of *AmphiHand*, the amphioxus ortholog of *Hand1* and *Hand2*, known to be expressed throughout the lateral plate mesoderm in zebrafish, chick, and mouse embryos (Charite et al., 2000; Deimling and Drysdale, 2009; Srivastava et al., 1995; Yelon et al., 2000). We observed the expression of *AmphiHand* during late neurula stages (8–13 somites), when the ventral mesoderm appears. At the 8/9-somite stage, *AmphiHand* transcripts were observed in the mid-ventral region of embryo (arrowheads in Fig. 1A). Subsequently, expression of *AmphiHand* expanded, reaching the caudal end of the embryo at the 12/13-somite stage (arrowheads in Fig. 1B–E). *AmphiHand* expression remained in the ventral mesoderm at least until the early larval stage (arrowheads in Fig. 1F).

We then examined the expression pattern of *AmphiNk2-tin* (Holland et al., 2003) to determine the distribution of the cardiac mesoderm. *Nkx2.5* in gnathostomes is reported to label the CM (Deimling and Drysdale, 2009). *AmphiNkx2-tin* expression was observed in the mid-ventral region at the 8/9-somite stage (arrowheads in Fig. 1G), and gradually expanded toward the caudal end of the body (arrowheads in Fig. 1G–K). *AmphiNk2-tin* expression persisted in the ventral mesoderm at least until early larval stage (arrowheads in Fig. 1L). Thus, *AmphiHand* and *AmphiNkx2-tin* were co-expressed in the ventral mesoderm at all stages examined, suggesting that the amphioxus ventral mesoderm posterior to the pharynx is not regionalized into cardiac mesoderm and posterior ventral mesoderm, at least as determined by expression of these markers.

To examine the distribution of the posterior ventral mesoderm in amphioxus, we initially searched for the amphioxus ortholog of *c-myb*, a gene used in zebrafish as a molecular marker for immature hematopoietic cells derived from the ventral side of the PLPM (Thompson et al., 1998). We identified only one ortholog of the *Myb* genes in the genome database (Putnam et al., 2008), which we named *AmphiMyb*. Transcripts of *AmphiMyb* were ubiquitously distributed in the body of amphioxus at both the 10/11-somite stage and early larval stages (Fig. S2). Thus, *AmphiMyb* is not suitable as a marker of the posterior ventral mesoderm in amphioxus.

We then examined whether the lateral plate mesoderm was regionalized into the CM and the PLPM in the lamprey embryo (Fig. 2). We examined the expression of *LjHandA*, the lamprey ortholog of *Hand1/2*, to determine the distribution of the lateral plate mesoderm. At stage 22, weak *LjHandA* signals were detected in the cardiac

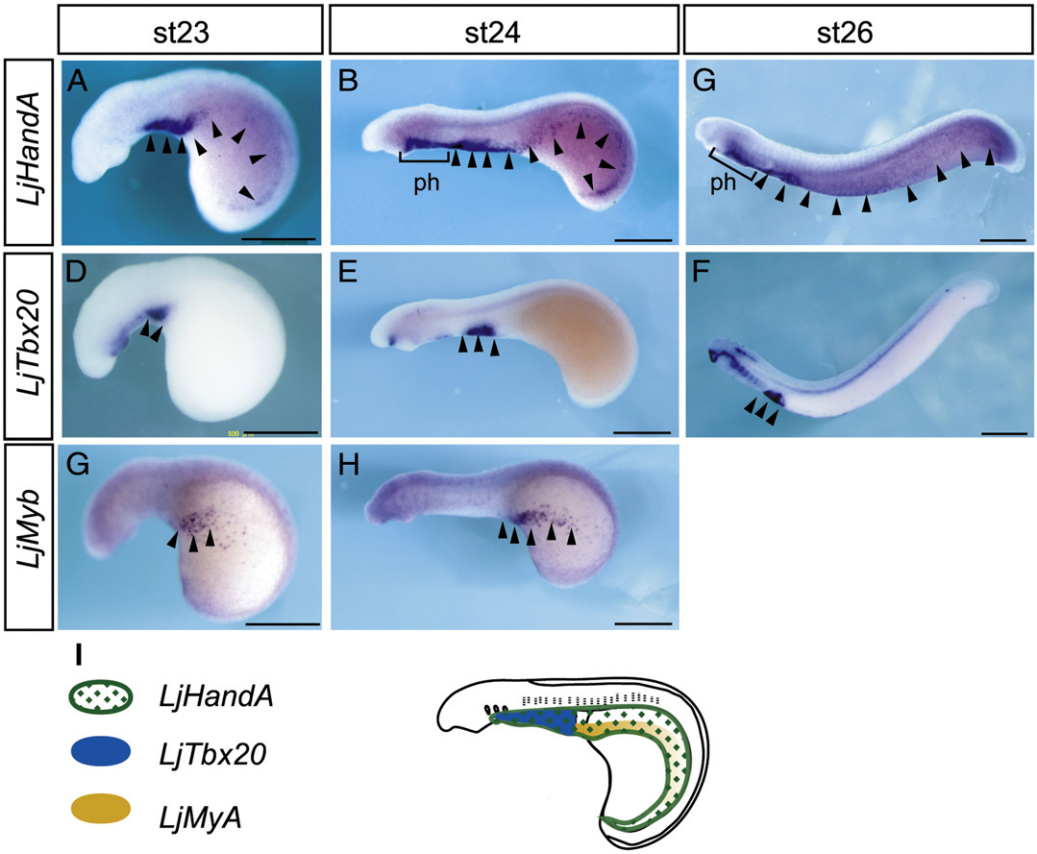


Fig. 2. Expression of *LjHandA*, *LjTbx20*, and *LjMyb* in the lateral plate mesoderm of lamprey embryos. (A–H) Lateral view of embryos at stages 23, 24, and 26. Anterior to the left, dorsal to the top. (A–C) Arrowheads indicate *LjHandA*-expressing cells in the lateral plate mesoderm. (D–F) Arrowheads indicate *LjTbx20*-expressing cardiac cells in the anterior lateral plate mesoderm. (G, H) Arrowheads indicate *LjMyb*-expressing immature hematopoietic cells within the posterior lateral plate mesoderm. ph, pharyngeal arch. Scale bars: 500 μm. (I) Schematic model of expression patterns in lamprey embryos. *LjHandA* signals (green dots) were observed throughout the lateral plate mesoderm, *LjTbx20*-expressing cells (light blue) were observed in the anterior part of the *LjHandA*-positive area, and *LjMyb*-expressing cells (light yellow) were seen in the region posterior to the *LjTbx20*-positive area.

mesoderm, and very faint signals were also seen in the population of cells in the lateral body wall (data not shown). By stage 23, intense *LjHandA* expression had appeared in the cardiac mesoderm. We also observed an *LjHandA*-expressing domain in the lateral plate mesoderm caudal to the presumptive heart field (arrowheads in Fig. 2A). At stage 24, strong expression of *LjHandA* was observed in the heart tube and also in cells of the lateral plate mesoderm caudal to the heart tube (arrowheads in Fig. 2B). The intense *LjHandA* expression in the heart remained until stage 26. We also observed *LjHandA*-expressing lateral plate mesoderm covering the yolk.

To examine the distribution of the CM in lamprey embryos, we examined the expression of *LjTbx20* (Kokubo et al., 2010), the lamprey ortholog of *Tbx20*, a gene expressed in heart fields of mouse and chick embryos (Kraus et al., 2001). As we previously showed (Kokubo et al., 2010), *LjTbx20* transcripts were first detected in the cardiac precursor at stage 21 (not shown), and continued to be expressed in the formed heart tube (arrowheads in Fig. 2D–F). Thus, in lamprey embryos, the CM occupies the anterior part of the *LjHandA*-expressing lateral plate mesoderm.

To clarify whether the *LjTbx20*-negative region of the lateral plate mesoderm represented the PLPM, we then examined the expression of *LjMyb* in the lamprey embryo as a molecular marker for the ventral side of the PLPM. *LjMyb* expression first appeared in cells of the lateral plate mesoderm caudal to the *LjTbx20*-positive region at stage 23 (arrowheads in Fig. 2G). Until stage 24, *LjMyb* continued to be expressed in the mesodermal cells caudal to the heart fields. The signal disappeared from the lateral plate mesodermal cells by stage 26, leaving only weak signals that were seen throughout the embryonic body (not shown). These latter signals probably reflect initiation of the blood circulation (Tahara, 1988). Taken together, our results suggest that the lateral plate mesoderm is regionalized into the CM and PLPM in lamprey embryos.

Expression of Hox in the lamprey lateral plate mesoderm

Hox gene expression in the lateral plate mesoderm is suspected to specify the positions along the anterior–posterior axis where limbs develop in chick embryos (Cohn et al., 1997; Nowicki and Burke, 2000). It has also been proposed that emergence of paired fins during vertebrate evolution was associated with appearance of staggered *Hox* expression boundaries in the lateral plate mesoderm (Cohn and Tickle, 1999). In zebrafish, chick, and mouse embryos, pectoral fins/forelimbs arise around the anterior border of *Hox5*- and *Hox6*-expressing regions in the lateral plate mesoderm (Becker et al., 1996; Nelson et al., 1996; Oliver et al., 1988; Waxman et al., 2008).

To investigate whether the lateral plate mesoderm of the lamprey expresses *Hox*, we examined *LjHox5i* and *LjHox6w* (Takio et al., 2004) expression in lamprey embryos (Fig. 3). In stage 21 embryos, *LjHox5i* and *LjHox6w* transcripts were found in the primitive paraxial mesoderm and the neural tube (arrowheads in Fig. 3A, D, G, J). At stage 22, the anterior border of *LjHox5i* expression in the lateral plate mesoderm was found at the level of somites 9–10 (arrowheads in Fig. 3B and E), whereas that of *LjHox6w* was at the level of somites 14–15 (arrowheads in Fig. 3H and K). In addition, expression of *LjHox5i* and *LjHox6w* was observed at the same levels in the paraxial mesoderm as in the lateral plate mesoderm (white arrowheads in Fig. 3B and H; also see Fig. S3). *LjHox5i* and *LjHox6w* continued to be expressed in the same regions of paraxial and lateral plate mesoderm until stage 23 (Fig. 3C, F, I). In addition, expression of *LjHox5i* was

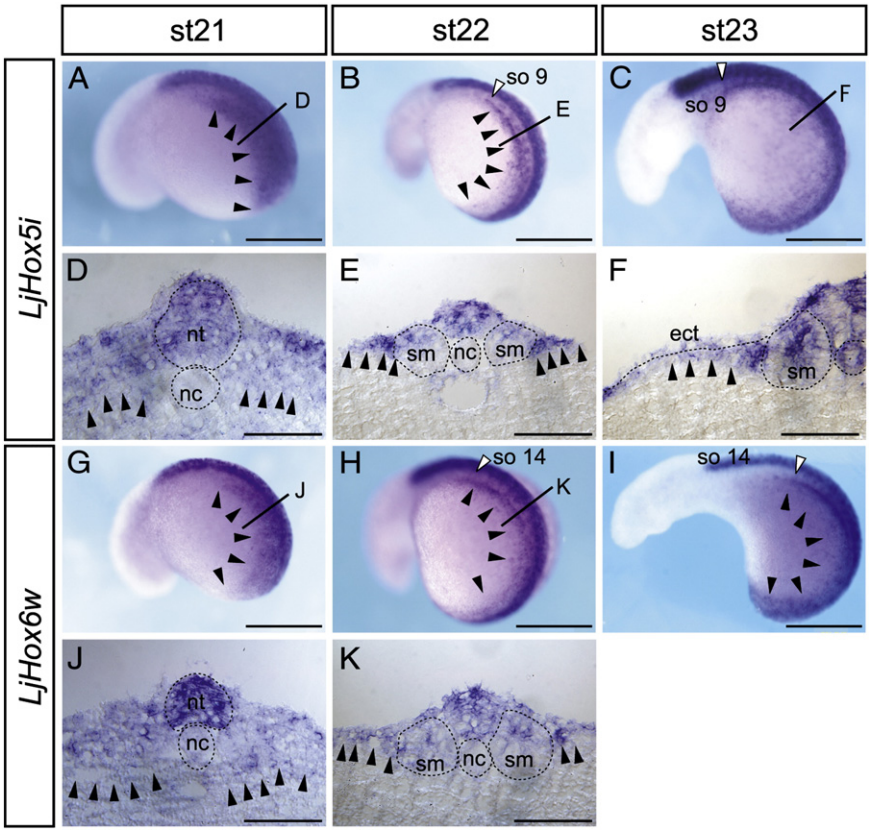


Fig. 3. Expression of *LjHox5i* and *LjHox6w* in the lateral plate mesoderm of lamprey embryos. (A–C, G–I) Lateral view of embryos at stages 21, 22, and 23. Anterior to the left, dorsal to the top. (D–F, J, K) Transverse sections through the lateral plate mesoderm where indicated in (A–C, G, H). Black arrowheads indicate *Hox* expression in the lateral plate mesoderm; white arrowheads indicate anterior borders of *Hox* expression in the paraxial mesoderm. ect, ectoderm; nc, notochord; sm, somatic mesoderm; so, somites. Scale bars: 500 μm (A–C, G-I), 100 μm (D–F, J, K).

detected in the overlying ectoderm. These results indicate that *Hox* genes are also likely to be involved in the specification of the lateral plate mesoderm in the lamprey.

Fin/limb initiation signals

Next, we examined whether the lamprey PLPM has acquired the expression domains of genes known to be involved in gnathostome limb initiation (Agarwal et al., 2003; Ahn et al., 2002; Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999; Takeuchi et al., 2003). We first examined the embryonic expression of *LjFgf8/17* (Shigetani et al., 2002), the lamprey ortholog of *Fgf8*. *Fgf8* encodes proteins that are secreted from the apical ectodermal ridge of fin/limb buds and promote fin/limb outgrowth (Crossley et al., 1996; Vogel et al., 1996). As previously reported (Shigetani et al., 2002), strong expression of *LjFgf8/17* was observed in the pharyngeal mesoderm as well as the midbrain–hindbrain boundary and the tail buds. We did not, however, detect any *LjFgf8/17* expression in the ectoderm overlying the PLPM at any stage examined (Fig. S4A–C).

In gnathostome limb/fin buds, ectodermal expression of *Fgf8* is induced by Fgf10 secreted from the mesoderm of fin/limb-forming fields (Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999). We isolated a lamprey ortholog of *Fgf10* called *LjFgf7/10/22* (Fig. S1) and examined its expression pattern (Fig. S4D–F). *LjFgf7/10/22* was

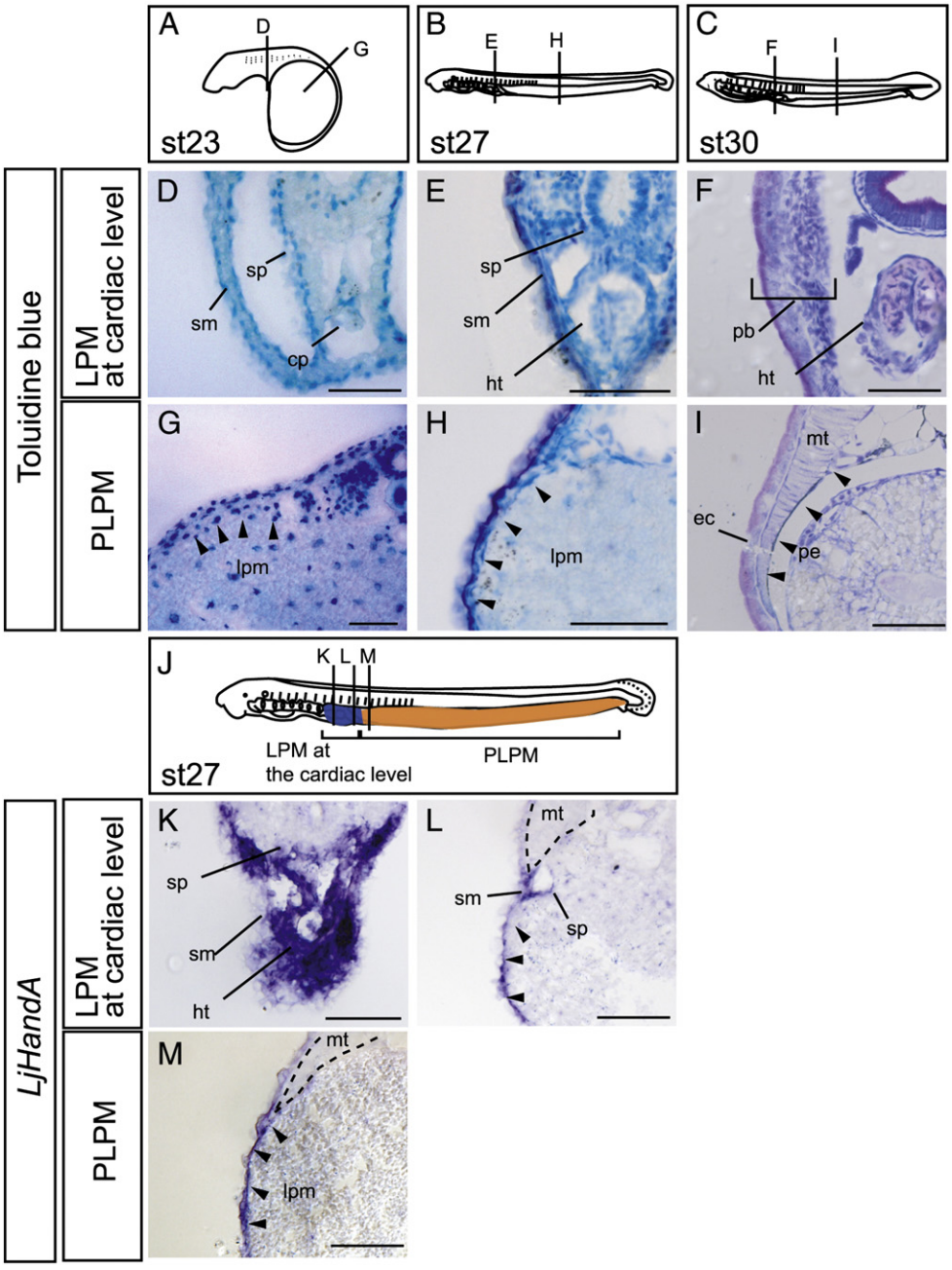


Fig. 4. Histological analysis of the lateral plate mesoderm in lamprey embryos. (A–C, J) Schematic diagrams of lamprey embryos at stages 23, 27, and 30. (D–I, K–M) Transverse sections through the lateral plate mesoderm at the cardiac level (D–F, K, L) and PLPM (G–I, M) where indicated in (A–C, J). (D–I) Toluidine blue staining. (K–M) Expression of *LjHandA*. The lateral plate mesoderm at the cardiac level was separated into somatic (sm) and splanchnic (sp) mesoderm at stages 23 (D) and 27 (E, K, L), and contributed to the formation of the pericardial body wall (pb) and the heart (ht) by stage 30 (F). The underdeveloped PLPM was not separated (arrowheads in H, M), and contributed to the peritoneal epithelium (pe, arrowheads in I). cp, cardiac progenitor cells; ec, ectoderm; ht, heart tube; lpm, lateral plate mesoderm; mt, myotome; pb, pericardial body wall; sm, somatic mesoderm; sp, splanchnic mesoderm. Scale bars: 50 μm.

detected in mandibular mesoderm, otic vesicles, and lips, similar to zebrafish and chick embryos (Kelly et al., 2001; Pirvola et al., 2000). However, no transcripts were detected in the posterior lateral plate mesoderm at any stage examined (Fig. S4D–F).

Since Tbx5 induces *Fgf10* expression in prospective limb-forming regions (Agarwal et al., 2003; Ahn et al., 2002; Takeuchi et al., 2003), we examined the expression of its lamprey ortholog, *LjTbx4/5* (Kokubo et al., 2010). As previously reported (Kokubo et al., 2010), *LjTbx4/5* expression was observed in the *LjTbx20*-expressing CM but not in the PLPM at any stage examined (Fig. S4G–I). These results suggest that the PLPM of lamprey embryos has not acquired expression of the fin/limb initiation signals *LjFgf8/17*, *LjFgf7/10/22*, and *LjTbx4/5*.

Subdivision of the lateral plate mesoderm into somatic and splanchnic layers

In chick and mouse embryos, limb buds are derived from the somatic layers of the lateral plate mesoderm. To examine whether the lamprey lateral plate mesoderm splits into somatic and splanchnic layers, lamprey embryos were sectioned and stained with toluidine blue (Fig. 4A–I). At stage 23, the lateral plate mesoderm at the cardiac level was separated into somatic and splanchnic layers (sm and sp in Fig. 4D), whereas the PLPM consisted of a mesenchymal layer two cells thick (arrowheads in Fig. 4G). At stage 27, the heart tube had formed from the splanchnic layer (sp and ht in Fig. 4E), whereas the single cell layer of the PLPM had expanded ventrally to cover the yolk (arrowheads in Fig. 4H) along the entire longitudinal axis (not shown). By stage 30, the pericardiac wall had formed from the somatic layers (pb in Fig. 4F), whereas the PLPM remained unseparated prior to the ammocoete stage (~stage 30; see pe in Fig. 4I).

To confirm these observations, we examined the expression of the lateral plate mesoderm marker *LjHandA* in transverse sections of lamprey embryos (Fig. 4J–M). As expected, *LjHandA* transcripts were observed in the heart tube as well as the somatic and splanchnic mesoderm (sm and sp in Fig. 4K). Interestingly, at the posterior end of the lateral plate mesoderm (at the cardiac level, at somite 11), delamination of the somatic and splanchnic layers was only observed at the dorsal corner (arrowheads in Fig. 4L). Furthermore, we did not detect any separation of mesodermal layers at the anterior end of the PLPM (level of somite 12, arrowheads in Fig. 4M) or at any other level along the anterior–posterior axis of the PLPM (not shown). These results suggest that the lamprey embryonic lateral plate mesoderm splits into somatic and splanchnic layers at the cardiac level, whereas cells of the PLPM seem to have contributed to formation of the epithelium of the peritoneal cavity.

To gain insight into how lateral plate mesoderm acquired the specification of somatic and splanchnic layers during evolution, we investigated the expression of orthologs of *Irx3* and *FoxF1* in amphioxus and lamprey embryos. In chick embryos, *FoxF1* is expressed throughout the lateral plate mesoderm prior to its subdivision. Subsequent to subdivision, *Irx3* expression appears in the somatic mesoderm and *FoxF1* expression becomes restricted to the splanchnic mesoderm (Funayama et al., 1999). In amphioxus, expression patterns of three orthologs of *Irx* genes – *BfIrxA*, *BfIrxB*, *and BfIrxC* – have been examined previously (Kaltenbach et al., 2009). Among them, only *BfIrxC* is expressed in the ventral mesoderm cells lining the coelom of amphioxus, and the *BfIrxC* transcripts are restricted to the pharyngeal ventral mesoderm (Kaltenbach et al., 2009). Expression of *AmphiFoxF*, the amphioxus ortholog of *FoxF*, has been examined at the

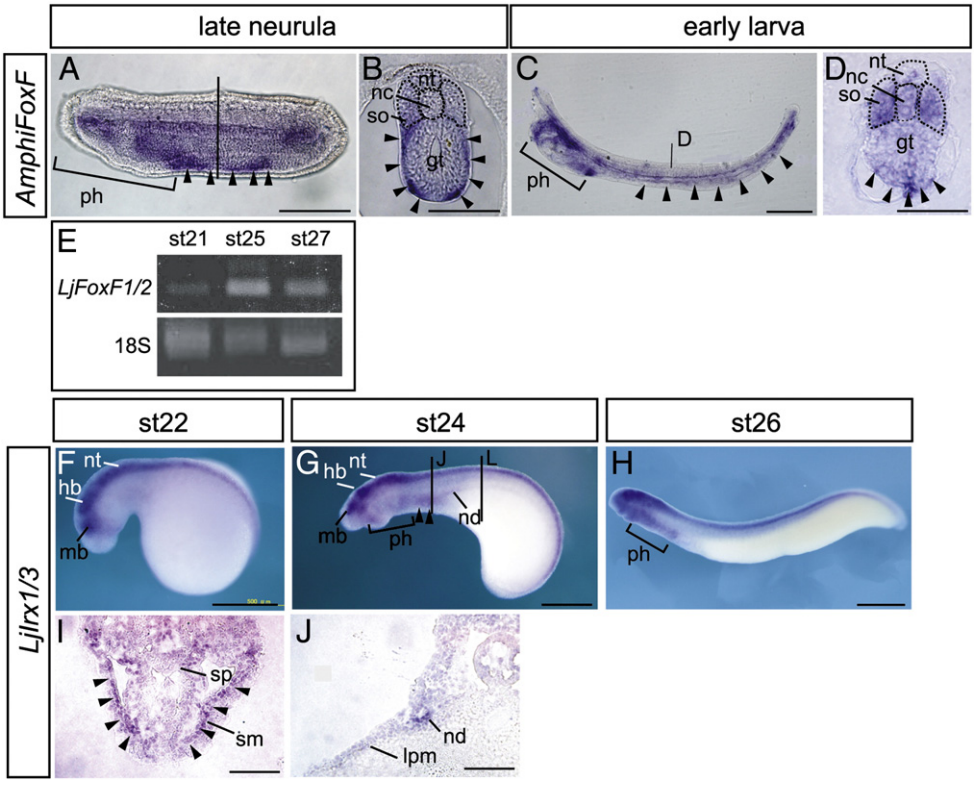


Fig. 5. Expression of *AmphiFoxF*, *LjFoxF1/2*, and *LjIrx1/3*. (A–D) Expression of *AmphiFoxF* at late neurula and early larval stages. (A, C) Lateral view. Anterior to the left, dorsal to the top. (B, D) Transverse sections at the level indicated in (A, C). (E) RT-PCR analysis of *LjFoxF1/2* expression in the mesoderm overlying the yolk of stage 21 embryos and in the PLPM of stage 25 and 27 embryos. (F–J) Expression of *LjIrx1/3* at stages 22, 24, and 26. (F–H) Lateral view. Anterior to the left, dorsal to the top. (I, J) Transverse sections at the level indicated in (G). *LjIrx1/3* signals are seen in the lateral plate mesoderm (arrowheads). Note that *LjIrx1/3* is expressed in the somatic layer at the cardiac level (I), but not in the PLPM (J). gt, gut; hb, hindbrain; lpm, lateral plate mesoderm; mb, midbrain; nc, notochord; nd, nephric duct; nt, neural tube; ph, pharynx; sm, somatic mesoderm; so, somites; sp, splanchnic mesoderm;. Scale bars: 25 μm in B, D; 100 μm in A, C, I, J; 500 μm in F–H.

early neurula stage and its expression in the ventral mesoderm was briefly described in the text (Mazet et al., 2006). To investigate whether *AmphiFoxF* is expressed in the ventral mesoderm, we examined the *AmphiFoxF* expression at late neurula and early larval stages. At late neurula stage, *AmphiFoxF* transcripts were observed throughout the ventral mesoderm, including the pharyngeal region (Fig. 5A). Transverse sections confirmed the distribution of *AmphiFoxF* in the ventral mesoderm (Fig. 5B). The *AmphiFoxF* expression in the ventral mesoderm remained until early larva stage (Fig. 5C, D). Thus, in amphioxus, *Irx* expression is restricted at the pharyngeal ventral mesoderm, whereas *FoxF* transcripts are distributed in the ventral mesoderm from the pharynx to the posterior end of the animal.

Finally, we isolated orthologs of *FoxF1/2* and *Irx1/3* from lampreys (Fig. S1D, E) and examined their expression patterns (Fig. 5E–J). The temporal expressions of *LjFoxF1/2* in the mesoderm overlying the yolk of stage 21 lamprey embryos, and the PLPM of stage 25 and 27 lamprey embryos were investigated by RT-PCR (Fig. 5E). At stage 21, transcripts of *LjFoxF1/2* were hardly detectable in the mesoderm overlying the yolk (Fig. 5E). By stage 25, strong expression of *LjFoxF1/2* was observed in the PLPM. *LjFoxF1/2* expression persisted in the PLPM until stage 27 (Fig. 5E). We then examined expression patterns of *LjIrx1/3* in lamprey embryos (Fig. 5F–J). At stage 22, *LjIrx1/3* expression was hardly detectable in the lateral plate mesoderm (Fig. 5F). By stage 24, intensified expression of *LjIrx1/3* was observed in the somatic layers at the cardiac level (Fig. 5G, I), but no expression of *LjIrx1/3* was detected in the PLPM (Fig. 5G, J). At stage 26, *LjIrx1/3* expression was reduced in somatic layers at the cardiac level (Fig. 5H). In addition, expression of *LjIrx1/3* was seen in the midbrain, hindbrain, neural tube, and pharynx (Fig. 5F–H), as seen in chick embryos (Funayama et al., 1999). Thus, in lampreys, the somatic layers at the cardiac level express *LjIrx1/3*, whereas the PLPM expresses *LjFoxF* but not *LjIrx1/3* at any stage examined.

Taken together, it appears that *FoxF* expression domains in the primitive ventral mesoderm were acquired prior to the lineage of amphioxus, and that *Irx3* expression domains were first acquired in the primitive ventral mesoderm of ancestral chordates at the pharyngeal level, and later in the somatic layers of the lateral plate mesoderm of primitive vertebrates.

Discussion

Establishment of the posterior lateral plate mesoderm

We show that the amphioxus ventral mesoderm posterior to the pharynx is not molecularly specified along the cardiac mesoderm and posterior ventral mesoderm. In support of our results, other recent works show that the amphioxus ortholog of *Tbx20* is expressed throughout the ventral mesoderm (Belgacem et al., 2011). On the other hand, expression of *Alx* and *Tbx1/10* is restricted to the pharyngeal ventral mesoderm of amphioxus embryos (Mahadevan et al., 2004; Meulemans and Bronner-Fraser, 2007), suggesting that regionalization between the pharyngeal ventral mesoderm and the ventral mesoderm posterior to the pharynx has already been established in the lineage of cephalochordates (Fig. 6A). Although the ventral mesoderm posterior to the pharynx is not regionalized into the cardiac mesoderm and the posterior ventral mesoderm, pharyngeal ventral mesoderm of cephalochordates can be regionalized along the anterior–posterior axis. We also show that, the lateral plate mesoderm in lamprey is regionalized into the CM and PLPM. Thus, the regionalization of the lateral plate mesoderm into the CM and the PLPM seems to have already occurred in the lineage of agnathans (Fig. 6B).

Recent studies in zebrafish and mouse embryos suggest that retinoic acid signaling delimits the adjacent heart fields and provides an environment that is permissive for forelimb induction (Waxman et al., 2008; Zhao et al., 2009). *Retinaldehyde dehydrogenase 2* (*Raldh2*)-deficient mice lacking retinoic acid synthesis show caudal expansion of the heart field and fail to undergo forelimb induction (Ryckebusch et al., 2008; Sirbu et al., 2008; Zhao et al., 2009). Similarly, zebrafish *raldh2* mutants lack pectoral fins (Begemann et al., 2001), and zebrafish embryos treated with the retinoic acid inhibitor 4-diethylaminobenzaldehyde (DEAB) show expanded heart fields and lack pectoral fins (Waxman et al., 2008). Taken together, these findings suggest that retinoic acid signaling may allow the lateral plate mesoderm to be regionalized into the CM and the PLPM by delimiting the CM.

During vertebrate evolution, such regionalization of the lateral plate mesoderm via retinoic acid signaling may have been a crucial step in acquiring fin-forming fields in the PLPM. Retinoic acid signaling plays pivotal roles during vertebrate development (Niederreither et al., 1999) and seems to be conserved during chordate development (Marletaz et al., 2006). *Raldh2* and *retinoic acid receptor* (*RAR*) genes have been identified in the genomes of both amphioxus and lampreys (Canestro et al., 2006). Furthermore, *RAR* and *Raldh2* transcripts have been observed in amphioxus and lamprey embryos, respectively (Castillo et al., 2010; Escriva et al., 2006). In addition, treatment of both amphioxus and lampreys with retinoic acid and retinoic acid inhibitors has been shown to affect anterior–posterior neural patterning (Murakami et al., 2004; Schubert et al., 2006). Thus, it is likely that the retinoic acid signaling pathway and its patterning roles were present in the common ancestor of vertebrates and amphioxus, although the distribution and function of retinoic acid in the ventral/lateral mesoderm of these animals remain elusive. We previously demonstrated that treatment of lampreys with a high concentration of retinoic acid induced loss of the heart (Kuratani et al., 1998). On the other hand, loss of Raldh2 function in gnathostomes leads to expansion of the heart fields and loss of forelimbs (Ryckebusch et al., 2008). These results also support our view that retinoic acid may have played critical roles for the regionalization of the lateral plate mesoderm into the CM and PLPM during evolution.

In zebrafish and mouse embryos, retinoic acid signaling seems to regionalize the lateral plate mesoderm by controlling the expression of *Hox* genes (Ryckebusch et al., 2008; Waxman et al., 2008). Inhibition of retinoic acid signaling in zebrafish with DEAB results in downregulation of *hoxb5b* in the lateral plate mesoderm, expansion of the heart fields, and the absence of pectoral fins (Waxman et al., 2008). Similarly, *Raldh2*-knockout mice show downregulation of *Hoxa1*, expansion of the heart fields, and failure to initiate forelimb bud formation (Ryckebusch et al., 2008). Thus, *Hox* expression in the lateral plate mesoderm seems to be critical for establishing the fin/limb-forming fields within the PLPM by delimiting the CM in the anterior region.

Hox gene expression was not detected in the segmented mesoderm of amphioxus (the progenitor of the ventral mesoderm; Holland et al., 1992; Wada et al., 1999), but was observed in the PLPM of lampreys, as in gnathostomes. These findings suggest that novel expression domains for *Hox* genes in the lateral plate mesoderm were acquired after the split of amphioxus and cyclostomes. We speculate that acquisition of novel expression domains for *Hox* genes in the lateral plate mesoderm may have been a crucial event leading to regionalization of the lateral plate mesoderm into the CM and PLPM.

Although we provide evidence that the amphioxus ventral mesoderm posterior to the pharynx is not regionalized into CM and posterior ventral mesoderm, we could not examine the distribution of the posterior ventral mesoderm, corresponding to the PLPM in vertebrates. *AmphiMyb* transcripts are not distributed exclusively in hematopoietic cells, but are ubiquitous throughout the body of amphioxus embryos and larvae. Ubiquitous expression of *AmphiMyb* might be related to the fact that amphioxus do not possess hemocytes (Moller and Philpott, 1973). Alternatively, PLPM-specific *Myb* might be unique to vertebrates. Vertebrate genomes contain three *Myb* genes: *A-Myb*, *B-Myb* and *c-Myb*. Phylogenetic analysis suggests that

A-Myb and *c-Myb* arose via recent gene duplication, which was preceded by the insertion of a novel transcriptional activation domain in the ancestral *A-Myb*/*c-Myb* gene, generated from the initial duplication of an ancestral *B-Myb*-like gene (Simon et al., 2002). Interestingly, *A-Myb* and *c-Myb* are expressed in the PLPM, but *B-Myb* is expressed ubiquitously (Sitzmann et al., 1996; Sitzmann et al., 1995). Thus, it is possible that amphioxus may not have acquired the PLPM-specific *Myb* in its genome. Without proper PLPM markers, it is not currently possible to distinguish whether the ventral mesoderm of ancestral chordates possessed the identity of only the CM or of both the CM and the PLPM. Future studies are therefore needed to investigate the expression of proper PLPM markers in amphioxus.

Expression of Hox genes in the lateral plate mesoderm

In this study, we report for the first time the expression of the *Hox* genes *LjHox5i* and *LjHox6w* in the lateral plate mesoderm of developing lamprey embryos. It is noteworthy that the anterior border of *LjHox5i* was located anterior to that of *LjHox6w* in the lamprey lateral plate mesoderm.

In zebrafish, chick, and mouse, the anterior appendages arise around the anterior limit of *Hox5* or *Hox6* expression in the lateral plate mesoderm (Becker et al., 1996; Nelson et al., 1996; Oliver et al., 1988; Waxman et al., 2008). In lampreys, the anterior borders of *LjHox5i* and *LjHox6w* expression in the lateral plate mesoderm were observed to be in the PLPM. It has been proposed that acquisition of staggered *Hox* boundaries in the lateral plate mesoderm was critical for the emergence of paired appendages during vertebrate evolution (Coates and Cohn, 1999). Taken together, these findings suggest that nested expression of *Hox* genes may pattern the lamprey lateral plate mesoderm along the anterior–posterior axis to position the pectoral fin-forming fields. Interestingly, nested expression of *LjHox* genes has also been observed in the central nervous system and pharyngeal arches of lamprey embryos (Takio et al., 2007; Takio et al., 2004). Although it is tempting to speculate about the collinearity of *Hox* genes in lampreys, we cannot draw any conclusions without complete information about the genomic linkage of *Hox* clusters in agnathan vertebrates. Elucidation of the complete structure of lamprey *Hox* clusters should provide insight into this problem.

We also observed nested expression of *LjHox5i* and *LjHox6w* in the somites of developing lamprey embryos. In our study, the anterior borders of *LjHox5i* and *LjHox6w* were seen at the level of somite 9 and 14, respectively. In gnathostomes, *Hox5* is expressed at the cervical level, whereas *Hox6* is expressed at the thoracic level (Burke et al., 1995). Furthermore, the anterior border of *Hox6* expression in the somites may be associated with the position of the brachial plexus and appendicular muscles (Burke et al., 1995). Similarly, lamprey *Hox* genes may position the levels of fin muscle precursors prior to the emergence of the pectoral fins. Although we have provided evidence for nested *Hox* expression in lamprey somites, the genomic evidence is too preliminary to advocate the collinearity of *Hox* in lampreys.

Development of subdivided somatic layers in the PLPM

In lampreys, we observed that the lateral plate mesoderm was subdivided into somatic and splanchnic layers at cardiac level by stage 23, whereas the PLPM remained a single, unseparated layer of cells prior to the ammocoete stage.

The limb buds of gnathostomes are derived from the somatic mesoderm of the PLPM and its overlying ectoderm. Unlike in tetrapods, subdivision of teleost PLPM has not been previously described. However, proliferation of the zebrafish PLPM was observed prior to the initiation of pectoral fin buds (Grandel and Schulte-Merker, 1998). Furthermore, *Hand2*-positive cells derived from the lateral plate mesoderm were observed around the developing gut at 24 h post-fertilization (Horne-Badovinac et al., 2003; Yin et al., 2010), suggesting that the zebrafish PLPM is likely to have separated during development. Without proper histological evidence, however, we cannot exclude the possibility that the unseparated PLPM contributes to the formation of both fins and gut muscles in teleost fishes.

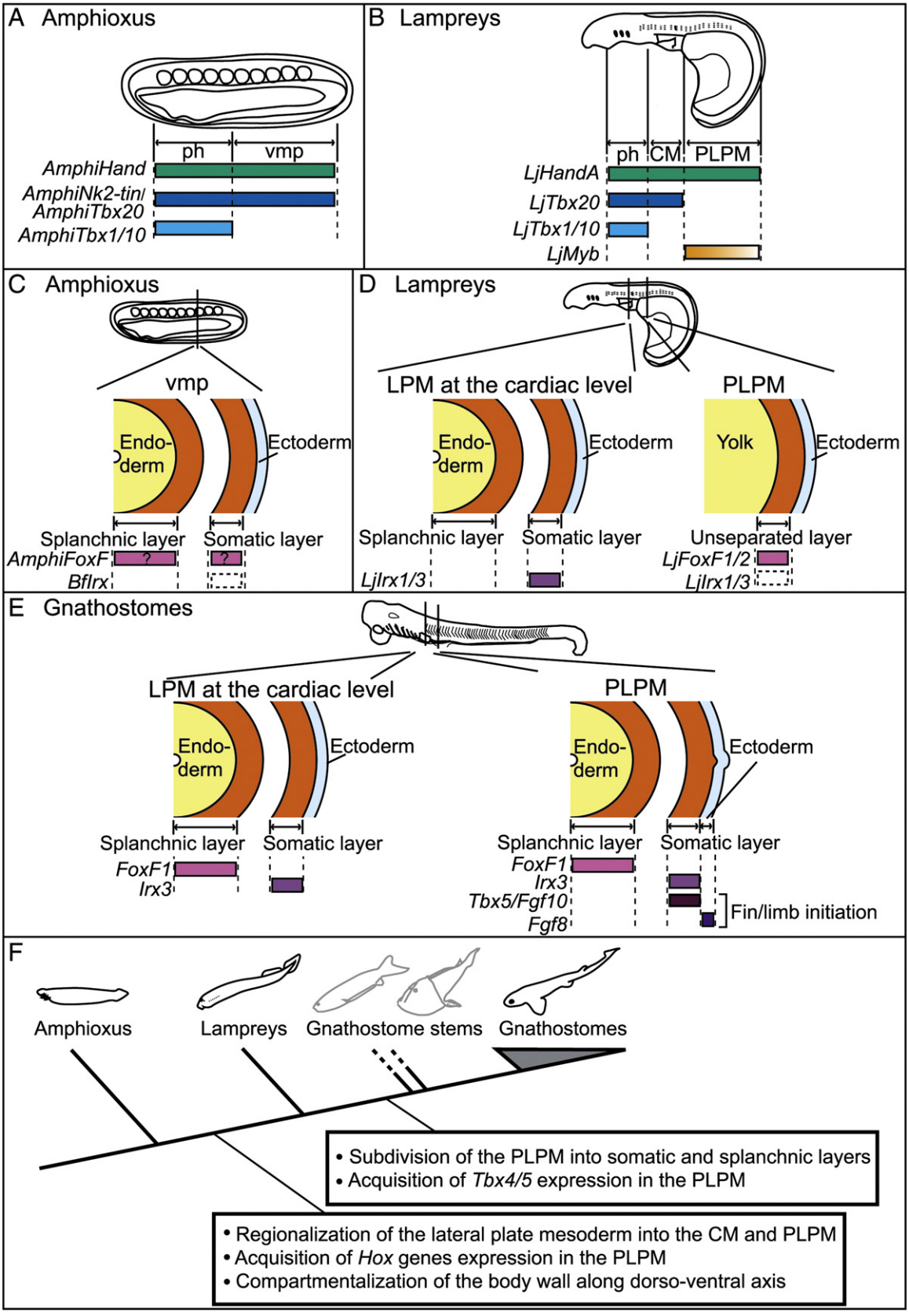
Proliferation and differentiation of the PLPM and formation of the coelom cavity are likely to be critical for the emergence of paired appendages. Further elucidation of the importance of PLPM differentiation for limb development is needed to address our hypothesis. In addition, although we suggest that cells derived from the PLPM contribute to the formation of the peritoneal epithelium and blood cells in lampreys, we cannot currently test this theory due to the reduced expression of molecular markers in differentiated cells. Lineage tracing studies of cells that arise from the PLPM of lampreys should provide evidence to resolve this question.

Evolution of the somatic and splanchnic mesoderm

In chick and mouse embryos, the lateral plate mesoderm splits into somatic and splanchnic layers. Prior to the subdivision, *FoxF* is expressed throughout the lateral plate mesoderm. Subsequent to the subdivision, *FoxF* expression becomes restricted to the splanchnic mesoderm and *Irx3* expression appears in the somatic mesoderm (Fig. 6E; Funayama et al., 1999). In contrast, the lamprey lateral plate mesoderm splits into somatic and splanchnic layers at the cardiac level, but not in the PLPM. Furthermore, *LjIrx1/3* expression was observed at the cardiac somatic layers, but not in the PLPM, although *LjFoxF* was expressed in the PLPM (Fig. 6D). More interestingly, the amphioxus ventral mesoderm, which expresses cardiac mesoderm markers, splits into somatic and splanchnic layers and expresses *AmphiFoxF* from the pharynx to the posterior end of the animal. However, the amphioxus *Irx* ortholog *BflrxC* is expressed in the anterior-most ventral mesoderm at the pharynx level of early larvae, but not in the ventral mesoderm posterior to the pharynx (Fig. 6C; Kaltenbach et al., 2009). These results provide a possible explanation for the evolution of somatic and splanchnic layers of the lateral plate mesoderm. Somatic and splanchnic layers of primitive ventral mesoderm of ancestral chordates probably correspond to the same

Fig. 6. Model for evolution of the ventral and lateral plate mesoderm. (A–E) Summary of expression of molecular markers in amphioxus (A, C), lampreys (B, D), and gnathostomes (E). (A, B) Ventral mesoderm of amphioxus (A) and lateral plate mesoderm of lampreys (B). Note that the ventral mesodermal marker *AmphiHand* and the CM markers *AmphiNk2-tin*/*AmphiTbx20* are co-expressed in the amphioxus ventral mesoderm, whereas the *LjHandA*-positive lamprey lateral plate mesoderm is regionalized into the *LjTbx20*-positive CM and the *LjMyb*-positive PLPM (see text for more details). *AmphiTbx20* expression after Holland et al. (2003); *AmphiTbx1/10* expression after Mahadevan et al. (2004); *LjTbx1/10* expression after Tiecke et al. (2007). ph, pharyngeal ventral mesoderm; vmp, ventral mesoderm posterior to the pharynx. (C–E) Somatic and splanchnic layers of amphioxus (C), lampreys (D), and gnathostomes (E). Schematic diagrams of cross-sections in the ventral mesoderm/lateral plate mesoderm at indicated levels. *FoxF* expression domains are already acquired in the amphioxus ventral mesoderm posterior to the pharynx, although it remains unclear whether they are in the splanchnic and/or somatic layers (question marks in C). *Irx3* expression domains are not acquired in the amphioxus ventral mesoderm posterior to the pharynx (dashed box in C), but are in the somatic layers of the lamprey LPM at the cardiac level. Furthermore, the *Irx3* expression domains are acquired in the gnathostome PLPM, but not in the unseparated lamprey PLPM. Acquisition of the *Irx3*-positive somatic layers in the PLPM seems to be a critical step for the subsequent development of paired fins/limbs (see text for more details). *Bflrx* expression after Kaltenbach et al. (2009). LPM, lateral plate mesoderm. (F) Model for acquisition of paired fins during evolution. In cephalochordates, the primitive lateral plate mesoderm/ventral mesoderm was not regionalized along the anterior–posterior axis. After the divergence from cephalochordates, the lateral plate mesoderm was regionalized into the CM and PLPM. The PLPM acquired novel expression domains for *Hox* genes. Overlying ectoderm was compartmentalized dorso-ventrally by ventral *Engrailed* expression. In stem gnathostomes, mesodermal cells in the PLPM proliferated, and the developed PLPM was subdivided into somatic and splanchnic layers. The PLPM acquired novel expression domains of *Tbx4/5*, leading to the emergence of the first paired fins (see text for more details). *Tbx4/5* model after Ruvinsky and Gibson-Brown (2000); nested *Hox* expression model after Coates and Cohn (1999); *Engrailed* model after Matsuura et al. (2008) and Tanaka (2009).

layers of vertebrate lateral plate mesoderm at the cardiac level. *FoxF*-positive lateral plate mesoderm may have been present in the common ancestor of amphioxus and vertebrates. After the divergence of ancestral chordates and vertebrates, *Irx3*-positive somatic mesoderm may have been acquired in the lateral plate mesoderm at the cardiac level. The *FoxF*-positive unseparated PLPM may also have been



acquired prior to the lineage of lampreys. The PLPM seems to have split into two layers after ancestral vertebrates diverged from lampreys and acquired the *Irx3*-positive somatic layers.

Although the somatic and splanchnic layers are morphologically similar between the amphioxus ventral mesoderm and the lateral plate mesoderm of gnathostomes we cannot exclude the possibility that they represent homoplastic structures. Developmentally, the amphioxus ventral mesodermal cells budding from the somites split into somatic and splanchnic layers while they grow downward on either side of the body (Kozmik et al., 2001; Holland et al., 2003), whereas the somatic and splanchnic layers of gnathostomes are separated from the single lateral plate mesodermal layer. Furthermore, in amphioxus, the ventral coelomic cavity lined by the somatic and splanchnic layers seems to function as a vascular system (Stach, 1998). These differences in the developmental process and functions of their derivatives may not automatically refute the homologies of these layers, however, our present data are not capable of homologizing these embryonic layers in chordates. This question will require further analyses and considerations in the future.

In the above connection, we have proposed that subdivision of the primitive PLPM into somatic and splanchnic layers would have occurred after the divergence between lampreys and gnathostomes. It is equally possible, however, that the primitive PLPM of ancestral vertebrates had already separated, and the unseparated PLPM appeared secondarily in the lineage either of lampreys or cyclostomes. Observation of PLPM development in hagfish embryos may provide key information to distinguish between such possibilities. Specification of the somatic mesoderm seems to be required for the formation of paired fins, and *Irx* expression in the somatic mesoderm may play an important role in this process. Future studies are needed to explore the function of *Irx* in developmental processes to understand the role of *Irx* for the acquisition of paired fins.

Model for the emergence of paired fins during vertebrate evolution

We propose a model for the evolutionary sequence that led to the emergence of paired fins in the lateral plate mesoderm of ancestral vertebrates (Fig. 6).

We showed that the amphioxus ventral mesoderm posterior to the pharynx was not regionalized into the cardiac mesoderm and posterior domain, whereas the lateral plate mesoderm of lampreys was compartmentalized into the CM and PLPM. It has been suggested that nested expression of *Hox* genes in the lateral plate mesoderm of gnathostomes delimits the cardiac fields and provides an environment for limb formation (Waxman et al., 2008; Zhao et al., 2009). Based on these findings, one would expect, in ancestral chordates, that the primitive ventral mesoderm posterior to the pharynx would not be regionalized into anterior and posterior regions. Subsequently, *Hox* genes acquired novel expression domains in the lateral plate mesoderm in agnathan lineages, leading to the regionalization of the lateral plate mesoderm into the CM and PLPM. Although we do not know whether initial *Hox* expression showed collinear patterns in the primitive PLPM of ancestral vertebrates, we suspect that the nested expression of *Hox* genes allowed the positioning of fin-forming fields in the PLPM, as previously proposed (Coates and Cohn, 1999).

Limbs are positioned at the dorso-ventral compartment of the body ectoderm. In gnathostome embryos, including dogfish, zebrafish, chick, and mouse, *Engrailed 1* is expressed in the ventral ectoderm of the body wall and subsequently compartmentalizes the underlying lateral plate mesoderm (Altabef et al., 1997; Laufer et al., 1997; Loomis et al., 1996; Rodriguez-Esteban et al., 1997; Tanaka et al., 2002; Tanaka et al., 1998). Interestingly, *Engrailed* expression was also observed in the ectoderm of the ventral compartment of the body wall of Japanese lamprey embryos (Matsuura et al., 2008). The fact that the body ectoderm of the limbless lampreys acquired *Engrailed* expression domains supports our previous view that the body ectoderm of ancestral agnathans may have been compartmentalized dorso-ventrally due to *Engrailed* expression (Tanaka et al., 2002). Epithelial expression of *Engrailed 1* in mouse was shown to localize the pigment cells to the dorsal side (Candille et al., 2004; Cygan et al., 1997). Thus, it is tempting to suggest that lamprey *Engrailed* expression also plays a role in this process. Interestingly, *Engrailed* appears in the ectoderm of lampreys concomitant with the onset of pigment cell migration (Tahara, 1988). Thus, we speculate that ectodermal *Engrailed* expression used in ancestral limbless agnathans for pigment localization may also have been utilized for novel functions such as fin positioning along the dorso-ventral axis, although the role of lamprey *Engrailed* remains unclear.

In this study, we showed that lamprey PLPM was not subdivided into somatic mesoderm (somatopleure) and splanchnic mesoderm (splanchnopleure), and had not acquired an *Irx3*-expression domain. Acquisition of *Irx3* expression in the PLPM of ancestral agnathans may have provided a permissive environment for emergence of fin-forming fields.

In the limb initiation phase, Tbx5 is required for induction of *Fgf10* expression in the mesoderm of forelimb-forming fields, and Fgf10 induces *Fgf8* expression in the overlying ectoderm (Agarwal et al., 2003; Ahn et al., 2002; Min et al., 1998; Ohuchi et al., 1997; Sekine et al., 1999; Takeuchi et al., 2003). On the other hand, expression of the lamprey orthologs *LjTbx4/5*, *LjFgf7/10/22*, and *LjFgf8/17* was not observed in the body wall of developing lamprey embryos. Furthermore, the ability of amphioxus *Tbx4/5* genes to induce limb formation has been shown in mouse transgenic lines (Minguillon et al., 2009). Thus, acquisition of novel *Tbx4/5* expression domains in the PLPM of ancestral agnathans must have been crucial for acquisition of paired fins, a view that was originally proposed by (Ruvinsky and Gibson-Brown, 2000). However, *Tbx4/5* is expressed in the ventral mesoderm of limbless amphioxus (Horton et al., 2008). Our work resolves this apparent contradiction. Because the ventral mesoderm of amphioxus is not regionalized into cardiac mesoderm and posterior ventral mesoderm, the expression of *Tbx4/5* in the ventral mesoderm may contribute only to heart differentiation. We propose that acquisition of paired fins may have required novel expression domains of limb initiation signals in the ancestral lateral plate mesoderm, as well as regionalization and patterning along its anterior–posterior axis, compartmentalization along its dorso-ventral axis, increased cell proliferation, and subdivision into somatic and splanchnic layers.

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